CHIMERIC $GABA_B$ RECEPTOR

The present invention provides a novel method to identify substances that are agonists of $GABA_B$ receptors, using a ^{3}H-GABA binding assay in recombinant $GABA_BR1a/R2$ receptor expressing cells.

BACKGROUND OF THE INVENTION

GABA (γ-amino-butyric acid) is the most widely distributed amino acid inhibitory neurotransmitter in the central nervous system (CNS) activating two distinct families of receptors; the ionotropic $GABA_A$ and $GABA_C$ receptors for fast synaptic transmissions, and the metabotropic $GABA_B$ receptors governing a slower synaptic transmission.

$GABA_B$ receptors are members of the superfamily of seven transmembrane G-protein coupled receptors that are coupled to neuronal K^+ or Ca^{2+} channels. Presynaptic $GABA_B$ receptor activation has generally been reported to result in the inhibition of Ca^{2+} conductance, leading to a decrease in the evoked release of neurotransmitters. Post-synaptically the major effect of $GABA_B$ receptor activation is to open potassium channels, to generate post-synaptic inhibitory potentials.

The expression of $GABA_B$ receptors is widely distributed in the mammalian neuronal axis, with particularly high levels in the molecular layer of the cerebellum, interpeduncular nucleus, frontal cortex, olfactory nuclei, thalamic nuclei, temporal cortex, raphe magnus and spinal cord. $GABA_B$ receptors are also present in the peripheral nervous system, both on sensory nerves and on parasympathetic nerves. Their ability to modulate these nerves give them potential as targets in disorders of the lung, GI tract and bladder (Belley et al., 1999, Biorg. Med. Chem. 7:2697-2704).

A large number of pharmacological activities have been attributed to $GABA_B$ receptor activation, such as for example, analgesia, hypothermia, catatonia, hypotension, reduction of memory consolidation and retention, and stimulation of insulin, growth hormone and glucagon release (see Bowery, 1989, Trends Pharmacol. Sci. 10:401-407 for a review). It is well accepted that $GABA_B$ receptor agonists and antagonists are pharmacologically useful in indications such as stiff man syndrome, gastroesophogeal reflux, neuropathic pain, incontinence and treatment of cough and cocaine addiction. For example, the $GABA_B$ receptor agonist baclofen has been shown to reduce transient lower esophagal sphincter relaxations (TLESR) and is accordingly useful in the treatment of reflux as most episodes of reflux occur during TLESR. However, the current $GABA_B$ receptor agonists, such as baclofen, are relatively non-

selective and show a variety of undesirable behavioural actions such as sedation and respiratory depression. It would be desirable to develop more $GABA_B$ receptor agonists with an improved selectivety and less of the aforementioned undesirable effects.

Current methods of drug discovery generally involve assessing the biological activity of tens or hundreds of thousands of compounds in order to identify a small number of those compounds having a desired activity against a particular target, i.e. High Throughput Screening (HTS). In a typical HTS related screen format, assays are performed in multi-well microplates, such as 96, 384 or 1536 well plates, putting certain constrains to the setup of the assay to be performed including the availability of the source materials (i.e membrane preparations of cells expressing the recombinant $GABA_B$ receptor). HTS related screens are preferably performed at room temperature with a single measurement for each of the compounds tested in the assay, requiring short cycle times, with a reproducible and reliable output.

Present *in vitro* screens to identify compounds as agonists of the $GABA_B$ receptor, either rely on natural, less abundant resources such as binding assays in rat brain membranes or consist of functional screening assays, such as for example Ca^{2+} responses, c-AMP responses and effects on Ca^{2+} and K^+ channels performed in cells expressing a recombinant $GABA_B$ receptor. In some of these functional assays the $GABA_B$ receptors may be co-expressed with G-proteins, e.g. $G\alpha 16$ or Gqi5 or the chimeric G-protein G αq-z5, increasing G-protein coupling (Bräuner-Osborne & Krogsgaard-Larsen, 1999, Br. J. Pharmacol. 128:1370-1374). However, a $GABA_B$ agonist binding assay that would further reduce the HTS cycle time and the resources for biochemicals such as recombinant proteins, is currently unavailable.

The present invention describes the development of a Chinese Hamster Ovary (CHO) cell line co-expressing the human $GABA_B$ receptor subunits $GABA_BR1a$ and $GABA_BR2$, which were surprisingly found to demonstrate agonist binding in radioligand binding experiments. In addition, the present inventors demonstrated that the $hGABA_BR1a/GABA_BR2$ CHO cell line has one high affinity and one low affinity agonist binding site in the recombinant expressed $GABA_B$ receptor. Hence the $hGABA_BR1a/GABA_BR2$ CHO cell line provided by the present invention not only allows compound screening, but also provides a useful tool to characterize the nature of the compound –receptor interaction.

SUMMARY OF THE INVENTION

The present invention provides an isolated $GABA_B$ receptor protein comprising at least one $GABA_BR1a$ subunit and at least one $GABA_BR2$ subunit, characterized in that said $GABA_B$ receptor has one high affinity agonist binding site and one low affinity agonist binding site. In particular the isolated recombinant $GABA_B$ receptor protein expressed by the $hGABA_BR1a/GABA_BR2$ CHO cell line deposited at the Belgian Coordinated Collections of Microorganisms (BCCM) as CHO-K1 h-GABA-b R1a/R2 clone on August 22, 2003 with the accession number LMBP 6046CB. It is thus an object of the present invention to provide the $hGABA_BR1a/GABA_BR2$ CHO cell line deposited at the Belgian Coordinated Collections of Microorganisms (BCCM) as CHO-K1 h-GABA-b R1a/R2 clone on August 22, 2003 with the accession number LMBP 6046CB.

The invention also provides the use of the aforementioned cell line in a method to identify $GABA_B$ receptor agonists using a functional or a binding assay. In particular in a radioligand-binding assay comprising the use of radiolabeled agonists such as for example ^{3}H-GABA or ^{3}H-baclofen.

The invention further provides a method to identify $GABA_B$ receptor agonists, comprising contacting the aforementioned cell line with a test compound and measuring the binding of said test compound to the $GABA_B$ receptor. In particular the method consists of a radioligand binding assay, comprising exposing the aforementioned cells to a labelled agonist of $GABA_B$ in the presence and absence of the test compound and measure the binding of the labelled ligand to the cells according to the invention, where if the amount of binding of the labelled ligand is less in the presence of the test compound, then the compound is a potential agonist of the $GABA_B$ receptor.

It is also an object of the present invention to provide a method to identify a high affinity $GABA_B$ receptor agonist, said method comprising contacting the aforementioned cells with the radiolabeled agonist selected from the group consisting of GABA, baclofen and 3-aminopropylphosphinic acid (3-APPA a.k.a APMPA), in the presence and absence of the test compound and measure the binding of the labelled ligand to the cells according to the invention, where if the amount of binding of the labelled ligand to the high affinity binding site is less in the presence of the test compound, then the compound is a potential high affinity agonist of the $GABA_B$ receptor.

Alternatively, the aforementioned binding assays are performed on cellular extracts, in particular cellular membrane preparations of the $hGABA_BR1a/GABA_BR2$ CHO cell line deposited at the Belgian Coordinated

Collections of Microorganisms (BCCM) as CHO-K1 h-GABA-b R1a/R2 clone on August 22, 2003 with the accession number LMBP 6046CB.

In another embodiment the present invention provides a method to identify a $GABA_B$ receptor agonist, said method comprising contacting the aforementioned cell line with a compound to be tested and determine whether the compound activates a $GABA_B$ receptor functional response in said cells. In particular the functional response consists of modulation of the activity of ion channels or of intracellular messengers as explained hereinafter.

This and further aspects of the present invention will be discussed in more detail hereinafter.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 GTPγ35S-binding upon stimulation of membranes by GABA expressed as the percentage of maximal GABA stimulation, in the presence and absence of the positive allosteric modulator CGP7930>

Figure 2 Displacement of ^{3}H-GABA by agonists (baclofen, GABA & APMPA) and antagonists (SCH50911 & CGP54626)

Figure 3 Reproducible agonist IC_{50} values (n=5) independent of membrane preparations.

Figure 4 Two sided ^{3}H-GABA agonist binding curve in the presence or absence of 10 μM CGP54626 (a) or JNJ 4309747-AAD (b).

DETAILED DESCRIPTION

For the purposes of describing the present invention: $GABA_BR1a$ or h $GABA_BR1a$ as used herein refers to the human $GABA_B$ receptor subunit known as $GABA_BR1a$ in Kaupmann et al, 1998, Proc. Natl. Acad. Sci. USA 95:14991-14996, the amino acid sequence (SEQ ID No.:2) of which can be found at GenBank Accession no. AJ225028, as well as to its mammalian orthologs. $GABA_BR1a$ also refers to other $GABA_B$ receptor subunits that have minor changes in amino acid sequence from those described

hereinbefore, provided those other $GABA_B$ receptor subunits have substantially the same biological activity as the subunits described hereinbefore. A $GABA_BR1a$ subunit has substantially the same biological activity if it has an amino acid sequence that is at least 80% identical to, preferably at least 95% identical to, more preferably at least 97% identical to, and most preferably at least 99% identical to SEQ ID No.: 2 and has a Kd or EC50 for GABA, $GABA_B$ receptor agonists such as for example baclofen and gabapentin or $GABA_B$ receptor antagonists such as for example CGP54626A, SCH 50911, saclofen and phaclofen, that is no more than 5-fold greater than the Kd or EC50 of a native $GABA_B$ receptor for GABA or the same $GABA_B$ receptor agonist or $GABA_B$ receptor antagonist.

$GABA_BR2$ as used herein refers to the human $GABA_B$ receptor subunit known as $GABA_BR2$ in White et al., 1998, Nature 396:679-682, the amino acid sequence (Seq Id NO.: 4) of which can be found at GenBank accession no. AF058795 as well as to its mammalian orthologs. $GABA_BR2$ also refers to other $GABA_B$ receptor subunits that have minor changes in amino acid sequence from those described hereinbefore, provided those other $GABA_B$ receptor subunits have substantially the same biological activity as the subunits described hereinbefore. A $GABA_BR2$ subunit has substantially the same biological activity if it has an amino acid sequence that is at least 80% identical to, preferably at least 95% identical to, more preferably at least 97% identical to, and most preferably at least 99% identical to SEQ ID No.: 4 and has in combination with a $GABA_BR1$ subunit a Kd or EC50 for GABA, $GABA_B$ receptor agonists such as for example baclofen and gabapentin or $GABA_B$ receptor antagonists such as for example CGP54626A, SCH 50911, saclofen and phaclofen, that is no more than 5-fold greater than the Kd or EC50 of a native $GABA_B$ receptor for GABA or the same $GABA_B$ receptor agonist or $GABA_B$ receptor antagonist.

The Kd and EC50 values of the native $GABA_B$ receptor is determined using the methods known to a person skilled in the art, in particular using competition binding studies on tissue preparations such as for example described in Cross & Horton, 1987 Eur.J.Pharmacol. 141(1): 159-162. Briefly, crude synaptic membranes are prepared by homogenisation of whole brain, centrifugation (30 000 xg, 20 min.) and extensive washing. Total binding is measured by incubation of the membranes with ^{3}H-GABA or ^{3}H-baclofen, while non-specific binding is measured in the presence of 100 μM baclofen. Upon removal of unbound ligand by filtration, filters are counted in a β-counter or a Topcount Harvester (Packhard). For competition experiments the binding occurs in the presence of increasing concentration of unlabeled compound.

It is thus an object of the present invention to provide an isolated $GABA_B$ receptor protein formed by at least one $GABA_BR1a$ and at least one $GABA_BR2$ subunit further characterized in that said isolated $GABA_B$ has both a high and a low affinity agonist binding site. In a further embodiment this isolated $GABA_B$ receptor is a functional $GABA_B$ receptor expressed by a cell, wherein said cell does not normally express the $GABA_B$ receptor. Suitable cells which are commercially available, include but are not limited to L-cells, HEK-293 cells, COS cells, CHO cells, HeLa cells and MRC cells, in particular CHO cells wherein the $GABA_B$ receptor protein comprises at least one $GABA_BR1a$ subunit encoded by the oligonucleotide sequence consisting of SEQ ID No.1 and at least one $GABA_BR2$ subunit encoded by the oligonucleotide sequence consisting of SEQ ID No.3. In a more particular embodiment the isolated $GABA_B$ receptor according to the invention, consists of the receptor protein expressed by the $hGABA_BR1a/GABA_BR2$ CHO cell line deposited at the Belgian Coordinated Collections of Microorganisms (BCCM) as CHO-K1 h-GABA-b R1a/R2 clone 20 on August 22, 2003 with accession number LMBP 6046CB.

"Functional $GABA_B$ receptor"refers to a $GABA_B$ receptor formed by co-expression of $GABA_BR2$ and $GABA_BR1a$ in a cell, wherein said cell does not normally express the $GABA_B$ receptor, most preferably resulting in a heterodimer of $GABA_BR2$ and $GABA_BR1a$, where the functional $GABA_B$ receptor mediates at least one functional response when exposed to the $GABA_B$ receptor agonist GABA. Examples of functional responses are : pigment aggregation in Xenopus melanophores, negative modulation of cAMP levels, coupling to inwardly rectifying potassium channels, mediation of late inhibitory postsynaptic potentials in neurons, increases in potassium conductance, decreases in calcium conductance, MAPKinase activation, extracellular pH acidification, and other functional responses typical of G-protein coupled receptors. One skilled in the art would be familiar with a variety of methods of measuring the functional responses of G-protein coupled receptors such as the $GABA_B$ receptor (see, e. g., Lerner, 1994, Trends Neurosci.17: 142-146 [changes in pigment distribution in melanophore cells]; Yokomizo et al., 1997, Nature 387: 620-624 [changes in cAMP or calcium concentration; chemotaxis]; Howard et al., 1996, Science 273: 974-977 [changes in membrane currents in Xenopus oocytes]; McKee et al., 1997, Mol. Endocrinol. 11: 415-423 [changes in calcium concentration measured using the aequorin assay]; Offermanns & Simon, 1995, J. Biol. Chem. 270: 15175-15180 [changes in inositol phosphate levels]). Depending upon the cells in which heteromers of $GABA_BR1a$ and $GABA_BR2$ are expressed, and thus the G-proteins with which the

functional $GABA_B$ receptor thus formed is coupled, certain of such methods may be appropriate for measuring the functional responses of such functional $GABA_B$ receptors. It is well within the competence of one skilled in the art to select the appropriate method of measuring functional responses for a given experimental system.

The term "compound", "test compound", "agent" or "candidate agent" as used herein can be any type of molecule, including for example, a peptide, a polynucleotide, or a small molecule that one whishes to examine for their activity as $GABA_B$ receptor agonist, and wherein said agent may provide a therapeutic advantage to the subject receiving it. The candidate agents can be administered to an individual by various routes, including, for example, orally or parenterally, such as intravenously, intramuscularly, subcutaneously, intraorbitally, intracapsularly, intraperitoneally, intrarectally, intracisternally or by passive or facilitated absorption through the skin, using for example a skin patch or transdermal iontophoresis, respectively. Furthermore the compound can be administered by injection, intubation or topically, the latter of which can be passive, for example, by direct application of an ointment, or active, for example, using a nasal spray or inhalant, in which case one component of the composition is an appropriate propellant. The route of administration of the compound will depend, in part, on the chemical structure of the compound. Peptides and polynucleotides, for example, are not particular useful when administered orally because they can be degraded in the digective tract. However, methods for chemically modifying peptides, for example rendering them less susceptible to degradation are well know and include for example, the use of D-amino acids, the use of domains based on peptidomimetics, or the use of a peptoid such as a vinylogous peptoid.

The agent used in the screening method may be used in a pharmaceutically acceptable carrier. See, e.g., *Remington's Pharmaceutical Sciences*, latest edition, by E.W. Martin Mack Pub. Co., Easton, PA, which discloses typical carriers and conventional methods of preparing pharmaceutical compositions that may be used in conjunction with the preparation of formulations of the agents and which is incorporated by reference herein.

Cells

As already outlined above, the present invention provides a cell line stably transfected with expression vectors that direct the expression of the $GABA_B$ receptor subunits $GABA_BR1a$ and $GABA_BR2$ as defined hereinbefore. In particular CHO cells transfected with said expression vectors. Such expression vectors are routinely

constructed in the art of molecular biology and may involve the use of plasmid DNA and appropriate initiators, promoters, enhancers and other elements, which may be necessary, and which are positioned in the correct orientation, in order to allow for protein expression. Generally, any system or vector suitable to maintain, propagate or express polynucleotides to produce a polypeptide in a host may be used. The appropriate nucleotide sequence, i.e. the polynucleotide sequences encoding either the human $GABA_BR1a$ or $GABA_BR2$ subunit as defined hereinbefore, may be inserted into an expression system by any of a variety of well-known and routine techniques such as for example those set forth in Current Protocols in Molecular Biology, Ausbel et al. eds., John Wiley & Sons, 1997.

In a particular embodiment the CHO cells according to the invention are cotransfected with the commercially available expression vectors pcDNA3.1 comprising the polynucleotide sequences encoding for human $GABA_BR1a$ (SEQ ID No.:1) and human $GABA_BR2$ (SEQ ID No.: 3) respectively. More preferably the present invention provides a $hGABA_BR1a/GABA_BR2$ CHO cell line deposited at the Belgian Coordinated Collections of Microorganisms (BCCM) as CHO-K1 h-GABA-b R1a/R2 clone 20 on August 22, 2003 with the accession number LMBP 6046CB. This cell line is characterized in that the functional $GABA_B$ receptor in this CHO cell line has both a low and a high affinity binding site for $GABA_B$ receptor agonist. Using the cell line according to the invention, will not only allow compound screening, but also provides a useful tool for the characterization of the nature of the compound-receptor interaction, i.e. does it interact with the low or high affinity agonist binding site of the $GABA_B$ receptor.

For further details in relation to the preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, see for example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press.

Assays

The present invention also provides an assay for a compound capable of interacting with the functional $GABA_B$ receptor of the present invention, which assay comprises: providing the $GABA_B$ receptor expressed by the $hGABA_BR1a/GABA_BR2$ CHO cell line of the present invention, contacting said receptor with a putative binding

compound; and determining whether said compound is able to interact with said receptor.

In one embodiment of the assay, the receptor or subunits of the receptor may be employed in a binding assay. Binding assays may be competitive or non-competitive. Such an assay can accommodate the rapid screening of a large number of compounds to determine which compounds, if any, are capable of binding to the polypeptides.

Within this context, the present invention provides a method to identify whether a test compound binds to an isolated $GABA_B$ receptor protein of the present invention, and is thus a potential agonist or antagonist of the $GABA_B$ receptor, said method comprising;

a) contacting cells expressing a functional $GABA_B$ receptor, wherein such cells do not normally express the $GABA_B$ receptor, with the test compound in the presence and absence of a compound known to bind the $GABA_B$ receptor, and

b) determine the binding of the test compound to the $GABA_B$ receptor using the compound known to bind to the $GABA_B$ receptor as a reference.

Binding of the test compound or of the compound known to bind to the $GABA_B$ receptor, hereinafter also referred to as reference compound, is assessed using art-known methods for the study of protein-ligand interactions. For example, such binding can be measured by employing a labeled substance or reference compound. The test compound or reference compound can be labeled in any convenient manner known in the art, *e.g.* radioactively, fluorescently or enzymatically. In a particular embodiment of the aforementioned method, the compound known to bind to the $GABA_B$ receptor, a.k.a. the reference compound is detectably labeled, and said label is used to determine the binding of the test compound to the $GABA_B$ receptor. Said reference compound being labeled using a radiolabel, a fluorescent label or an enzymatic label, more preferably a radiolabel. In a more particular embodiment, the present invention provides a method to identify whether a test compound binds to an isolated $GABA_B$ receptor protein, said method comprising the use of a compound known to bind to the $GABA_B$ receptor, wherein said reference compound is selected from the group consisting of ^{3}H-GABA, ^{3}H-baclofen, ^{3}H-3-APPA, ^{3}H-CGP542626 and ^{3}H-SCH50911.

Subsequently, more detailed assays can be carried out with those compounds found to bind, to further determine whether such compounds act as agonists or antagonists of the polypeptides of the invention.

Thus, in a further embodiment the present invention provides a method to identify $GABA_B$ receptor agonists said method comprising,
a) exposing cells expressing a functional $GABA_B$ receptor, wherein such cells do not normally express the $GABA_B$ receptor, to a labeled agonists of $GABA_B$ in the presence and absence of the test compound, and
b) determine the binding of the labeled agonist to said cells,
where if the amount of binding of the labeled agonist is less in the presence of the test compound, then the compound is a potential agonist of the $GABA_B$ receptor. As already specified for the general binding assay above, the binding of the $GABA_B$ receptor agonists is assessed using art-known methods for the study of protein-ligand interactions. The label is generally selected from a radioactive label, a fluorescent label or an enzymatic label, in particular a radiolabel wherein the agonist is selected from the group consisting of ^{3}H-GABA, ^{3}H-baclofen and ^{3}H-3-APPA.

Similarly, the present invention provides a method to identify $GABA_B$ receptor antagonists said method comprising,
a) exposing cells expressing a functional $GABA_B$ receptor, wherein said cells do not normally express the $GABA_B$ receptor, to a labeled antagonist of $GABA_B$ in the presence and absence of the test compound, and
b) determine the binding of the labeled antagonist to said cells,
where if the amount of binding of the labeled antagonist is less in the presence of the test compound, then the compound is a potential antagonist of the $GABA_B$ receptor. As already specified for the general binding assay above, the binding of the $GABA_B$ receptor antagonists is assessed using art-known methods for the study of protein-ligand interactions. The label is generally selected from a radioactive label, a fluorescent label or an enzymatic label, in particular a radiolabel wherein the antagonist is selected from the group consisting of ^{3}H-CGP542626 and ^{3}H-SCH50911.

In an alternative embodiment of the present invention, the aforementioned binding assays are performed on a cellular composition, i.e a cellular extract, a cell fraction or cell organels comprising a $GABA_B$ receptor as defined hereinbefore. More in particular, the aforementioned binding assays are performed on a cellular composition, i.e. a cellular extract, a cell fraction or cell organels comprising a $GABA_B$ receptor as defined hereinbefore, wherein said cellular composition, i.e. cellular extract, cell fraction or cell organels, is obtained from cells expressing a functional $GABA_B$ receptor, wherein said cells do not normally express the $GABA_B$ receptor. More preferably, the cellular composition, i.e. cellular extract, cell fraction or cell organels, is

obtained from the hGABA$_B$R1a/GABA$_B$R2 CHO cell line deposited at the Belgian Coordinated Collections of Microorganisms (BCCM) as CHO-K1 h-GABA-b R1a/R2 clone 20 on August 22, 2003 with the accession number LMBP 6046CB.

It is accordingly, an object of the present invention to provide a method for identifying a compound as a GABA$_B$ receptor agonist or antagonist, said method comprising;

a) administering the compound to a cellular composition of cells expressing a functional GABA$_B$ receptor, wherein said cells do not normally express the GABA$_B$ receptor, in the presence of a detectably labeled agonist or antagonist of the GABA$_B$ receptor; and

b) determine the binding of the labeled agonist or antagonist to said cellular composition,

where if the amount of binding of the labeled agonist or antagonist is less in the presence of the test compound, then the compound is a potential agonist respectively antagonist of the GABA$_B$ receptor.

As already specified for the general binding assay above, the binding of the GABA$_B$ receptor agonist or antagonist is assessed using art-known methods for the study of protein-ligand interactions. The label is generally selected from a radioactive label, a fluorescent label or an enzymatic label, in particular a radiolabel wherein the agonist is selected from the group consisting of ^{3}H-GABA, ^{3}H-baclofen and ^{3}H-3-APPA and the antogonist is selected from the group consisting of ^{3}H-CGP542626 and ^{3}H-SCH50911. In a more specific embodiment the aforementioned binding assays are performed on a cellular composition consisting of the membrane fraction of cells according to the invention, in particular on membrane fractions of the hGABA$_B$R1a/GABA$_B$R2 CHO cell line deposited at the Belgian Coordinated Collections of Microorganisms (BCCM) as CHO-K1 h-GABA-b R1a/R2 clone 20 on August 22, 2003 with the accession number LMBP 6046CB, using one or more of the aforementioned radiolabeled agonsist and/or antagonists.

In a further embodiment the present invention provides a functional assay for identifying compounds that modulate the GABA$_B$-recepor activity in the cells according to the invention. Such an assay is conducted using the cells of the present invention, i.e. cotranfected with the human GABA$_B$R1a and human GABA$_B$R2 subunits. The cells are contacted with at least one reference compound wherein the ability of said compound to modulate the GABA$_B$-receptor activity is known. Thereafter, the cells are contacted with a test compound and determined whether said test compound modulates the activity of the GABA$_B$ receptor compared to the reference compound. A "reference

compound" as used herein refers to a compound that is known to bind and/or to modulate the $GABA_B$ receptor activity.

A compound or a signal that "modulates the activity" of a polypeptide of the invention refers to a compound or a signal that alters the activity of the polypeptide so that it behaves differently in the presence of the compound or signal than in the absence of the compound or signal. Compounds affecting modulation include agonists and antagonists. An agonist of the $GABA_B$ receptor encompasses a compound such as GABA, baclofen and 3 - APPA which activates $GABA_B$ receptor function. Alternatively, an antagonist includes a compound that interferes with $GABA_B$ receptor function. Typically, the effect of an antagonist is observed as a blocking of agonist-induced receptor activation. Antagonists include competitive as well as non-competitive antagonists. A competitive antagonist (or competitive blocker) interacts with or near the site specific for agonist binding. A non-competitive antagonist or blocker inactivates the function of the receptor by interacting with a site other than the agonist interaction site.

In one embodiment the present invention provides a method for identifying compounds that have the capability to modulate $GABA_B$ receptor activity, said method comprising;
a) contacting cells expressing a functional $GABA_B$ receptor, wherein said cells do not normally express a functional $GABA_B$ receptor, with at least one reference compound, under conditions permitting the activation of the $GABA_B$ receptor;
b) contacting the cells of step a) with a test compound, under conditions permitting the activation of the $GABA_B$ receptor, and
c) determine whether said test compound modulates the $GABA_B$ receptor activity compared to the reference compound.

Methods to determine the capability of a compound to modulate the $GABA_B$ receptor activity are based on the variety of assays available to determine the functional response of G-protein coupled receptors (see above) and in particular on assays to determine the changes in potassium currents, changes in calcium concentration, changes in cAMP and changes in GTPγS binding. Conditions permitting the activation of the $GABA_B$ receptor generally known in the art, for example in case of antagonist screening these conditions comprise the presence of a $GABA_B$ receptor agonist in the assay system. Typical $GABA_B$ receptor agonists used in these activity assays are GABA, baclofen or 3-APPA. More particular in the GTPγS assay as outlined herein below, GABA is used

to activate the $GABA_B$ receptor in order to assess the capability of a test compound to inactivate the $GABA_B$ receptor protein.

In the aforementioned assay an increase of GTPγS binding in the presence of the test compound is an indication that the compound activates the $GABA_B$ receptor activity, and accordingly that said test compound is a potential agonist of the $GABA_B$ receptor protein. A decrease of GTPγS binding in the presence of the test compound is an indication that the compound inactivates the $GABA_B$ receptor protein and accordingly that said test compound is a potential antagonist of the $GABA_B$ receptor protein.

Particularly preferred types of assays include binding assays and functional assays which may be performed as follows:

Binding assays

Over-expression of the $GABA_B$ receptor expressed by the $hGABA_BR1a/GABA_BR2$ CHO cell line of the present invention may be used to produce membrane preparations bearing said receptor (referred to in this section as $GABA_B$ binding receptor for convenience) for ligand binding studies. These membrane preparations can be used in conventional filter-binding assays (eg. Using Brandel filter assay equipment) or in high throughput Scintillation Proximity type binding assays (SPA and Cytostar-T flashplate technology; Amersham Pharmacia Biotech) to detect binding of radio-labelled $GABA_B$ ligands (including ^{3}H-GABA, ^{3}H-baclofen, ^{3}H-3-APPA, ^{3}H-CGP542626, ^{3}H-SCH50911) and displacement of such radio-ligands by competitors for the binding site. Radioactivity can be measured with Packard Topcount, or similar instrumentation, capable of making rapid measurements from 96-, 384-, 1536- microtitre well formats. SPA/Cytostar-T technology is particularly amenable to high throughput screening and therefore this technology is suitable to use as a screen for compounds able to displace standard ligands.

Another approach to study binding of ligands to $GABA_B$ binding receptor protein in an environment approximating the native situation makes use of a surface plasmon resonance effect exploited by the Biacore instrument (Biacore). $GABA_B$ binding receptor in membrane preparations or whole cells could be attached to the biosensor chip of a Biacore and binding of ligands examined in the presence and absence of compounds to identify competitors of the binding site.

Functional assays

Since $GABA_B$ receptors belong to the family G-protein coupled receptors that are coupled to GIRK (inward rectifying potassium channels), potassium ion flux should result on activation of these receptors. This flux of ions may be measured in real time using a variety of techniques to determine the agonistic or antagonistic effects of particular compounds. Therefore, recombinant $GABA_B$ binding receptor proteins expressed in the cell lines of the present invention can be characterised using whole cell and single channel electrophysiology to determine the mechanism of action of compounds of interest. Electrophysiological screening, for compounds active at $GABA_B$ binding receptor proteins, may be performed using conventional electrophysiological techniques and when they become available, novel high throughput methods currently under development.

Given the presynaptic effect of $GABA_B$ receptor activation on Ca^{2+} channels, in an alternative functional screen the modulatory effect of a compound is assessed through the changes in intracellular calcium. Calcium fluxes are measurable using several ion-sensitive fluorescent dyes, including fluo-3, fluo-4, fluo-5N, fura red and other similar probes from suppliers including Molecular Probes. The inhibition of calcium influx as a result of $GABA_B$ receptor activation can thus be characterised in real time, using fluorometric and fluorescence imaging techniques, including fluorescence microscopy with or without laser confocal methods combined with image analysis algorithms.

Another approach is a high throughput screening assay for compounds active as either agonists or modulators which affect calcium transients. This assay is based around an instrument called a **FL**uorescence **I**maging **P**late **R**eader ((FLIPR®), Molecular Devices Corporation). In its most common configuration, it excites and measures fluorescence emitted by fluorescein-based dyes. It uses an argon-ion laser to produce high power excitation at 488 nm of a fluorophore, a system of optics to rapidly scan the over the bottom of a 96-/384-well plate and a sensitive, cooled CCD camera to capture the emitted fluorescence. It also contains a 96-/384-well pipetting head allowing the instrument to deliver solutions of test agents into the wells of a 96-/384-well plate. The FLIPR assay is designed to measure fluorescence signals from populations of cells before, during and after addition of compounds, in real time, from all 96-/384-wells simultaneously. The FLIPR assay may be used to screen for and characterise compounds functionally active at the $hGABA_BR1a/GABA_BR2$ CHO cell line.

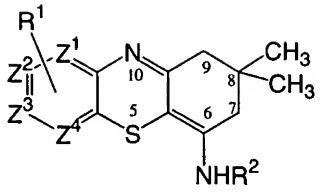
A high throughput screening assay, specifically usefull to identify $GABA_B$ agonists could consist of an arrangement wherein $hGABA_BR1a/GABA_BR2$ CHO cells, are loaded with an appropriate fluorescent dye, incubated with a test compound and after sufficient time to allow interaction (8 – 24 hours, typically 12-24 hours, in particular 24 hours.) the change in relative fluorescence units measured using an automated fluorescence plate reader such as FLIPR or Ascent Fluoroskan (commercially available from Thermo Labsystems, Brussel, Belgium).

In a further embodiment the functional assay is based on the change in GTPγS binding to the $GABA_B$ binding receptor. In particular using a competion bindig assay to determine the displacement of radiolabelled GTPγS. In general, this method to identify $GABA_B$-receptor agonists comprises preparing a membrane fraction from cells expressing the $hGABA_BR1a/GABA_BR2$ heterodimer af the present invention, contacting said membrane preparations with the compound to be tested in the presence of radiolabelled GTPγS, under conditions permitting the activation of the $GABA_B$ receptor, and detecting GTPγS binding to the membrane fraction. An increase in GTPγS binding in the presence of the compound is an indication that the compound activates the $hGABA_BR1a/GABA_BR2$ receptor. A decrease in GTPγS binding in the presence of the compound is an indication that the compound inactivates the $hGABA_BR1a/GABA_BR2$ receptor. Preferably this GTPγS binding assay is performed on membrane fractions obtained from the $hGABA_BR1a/GABA_BR2$ CHO cell line deposited at the Belgian Coordinated Collections of Microorganisms (BCCM) as CHO-K1 h-GABA-b R1a/R2 clone 20 on August 22, 2003 with the accession number LMBP 6046CB. Further, the conditions permitting the activation of the $GABA_B$ receptor comprise the presence of a $GABA_B$ receptor agonist, such as for example GABA, baclofen and 3-APPA in the assay system. In particular GABA.

This and other functional screening assays will be provided in the examples hereinafter.

$GABA_B$ receptor agonists

In a further aspect the present invention provides $GABA_B$ receptor agonists identified using one of the aforementioned screening assays wherein said $GABA_B$ receptor agonists are represented by the compounds of formula (I)



(I)

the *N*-oxide forms, the pharmaceutically acceptable addition salts and the stereochemically isomeric forms thereof, wherein

$=Z^1-Z^2=Z^3-Z^4=$ represents a divalent radical selected from the group consisting of =N-CH=CH-N= (a), =N-CH=N-CH= (b), =CH-N=CH-N= (c) =CH-CH=CH-CH= (d), =N-CH=CH-CH= (e), =CH-N=CH-CH= (f), =CH-CH=N-CH= (g) and =CH-CH=CH-N= (h);

R^1 represents hydrogen, halo, hydroxyl, cyano, C_{1-6}alkyl, CF_3, amino or mono- or di(C_{1-4}alkyl)amino;

R^2 represents hydrogen, C_{1-6}alkyl or hydroxycarbonyl-C_{1-6}alkyl-.

In particular those compounds of formula (I) wherein one or more of the following restrictions apply;

(i) $=Z^1-Z^2=Z^3-Z^4=$ represents a divalent radical selected from the group consisting of =N-CH=CH-N= (a), =N-CH=N-CH= (b), =CH-N=CH-N= (c) and =CH-CH=CH-CH= (d);

(ii) R^1 represents halo, amino or mono- or di(C_{1-4}alkyl)amino;

(iii) R^2 represents butyric acid

Also of interest are those compounds of formula (I) wherein;

(i) R^1 is attached at position Z^1; and/or

(ii) $=Z^1-Z^2=Z^3-Z^4=$ represents (a), (b) or (d), more preferably $=Z^1-Z^2=Z^3-Z^4=$ represents (d).

As used in the foregoing definitions and hereinafter, halo is generic to fluoro, chloro, bromo and iodo; C_{1-4}alkyl defines straight and branched chain saturated hydrocarbon radicals having from 1 to 4 carbon atoms such as, for example, methyl, ethyl, propyl, butyl, 1-methylethyl, 2-methylpropyl, 2,2-dimethylethyl and the like; C_{1-6}alkyl defines straight and branched chain saturated hydrocarbon radicals having from 1 to 6 carbon atoms such as, for example, pentyl, hexyl, 3-methylnutyl, 2-methylpentyl and the like.

The pharmaceutically acceptable addition salts as mentioned hereinabove are meant to comprise the therapeutically active non-toxic acid addition salt forms, which the compounds of formula (I), are able to form. The latter can conveniently be obtained by treating the base form with such appropriate acid. Appropriate acids comprise, for example, inorganic acids such as hydrohalic acids, e.g. hydrochloric or hydrobromic acid; sulfuric; nitric; phosphoric and the like acids; or organic acids such as, for example, acetic, propanoic, hydroxyacetic, lactic, pyruvic, oxalic, malonic, succinic (i.e. butanedioic acid), maleic, fumaric, malic, tartaric, citric, methanesulfonic, ethanesulfonic, benzenesulfonic, *p*-toluenesulfonic, cyclamic, salicylic, *p*-aminosalicylic, pamoic and the like acids.

The pharmaceutically acceptable addition salts as mentioned hereinabove are meant to comprise the therapeutically active non-toxic base addition salt forms which the compounds of formula (I), are able to form. Examples of such base addition salt forms are, for example, the sodium, potassium, calcium salts, and also the salts with pharmaceutically acceptable amines such as, for example, ammonia, alkylamines, benzathine, *N*-methyl-D-glucamine, hydrabamine, amino acids, e.g. arginine, lysine.

Conversely said salt forms can be converted by treatment with an appropriate base or acid into the free acid or base form.

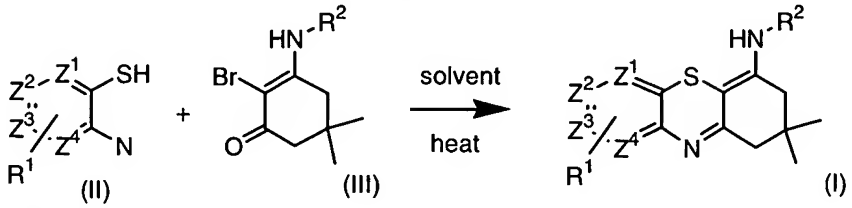
The term addition salt as used hereinabove also comprises the solvates which the compounds of formula (I), as well as the salts thereof, are able to form. Such solvates are for example hydrates, alcoholates and the like.

The term stereochemically isomeric forms as used hereinbefore defines the possible different isomeric as well as conformational forms which the compounds of formula (I), may possess. Unless otherwise mentioned or indicated, the chemical designation of compounds denotes the mixture of all possible stereochemically and conformationally isomeric forms, said mixtures containing all diastereomers, enantiomers and/or conformers of the basic molecular structure. All stereochemically isomeric forms of the compounds of formula (I), both in pure form or in admixture with each other are intended to be embraced within the scope of the present invention.

The *N*-oxide forms of the compounds of formula (I), are meant to comprise those compounds of formula (I) wherein one or several nitrogen atoms are oxidized to the so-called *N*-oxide.

The 7,8-dihydro-phenothiazine derivatives of the present invention are generally prepared as described by Nemeryuk M.P. et al., Khimiko-Farmatsevticheskii Zhurnal (1985), 19(8), 964-968. In brief, the known ortho-amino substituted (hetero)arene-thiols (II), are condensed with an appropriate 2-bromo-5,5-dimethyl-3-oxo-cyclohex-1-enylamino derivative (III), by heating the two reactants in a suitable solvent, such as ethanol or N-methylpyrrolidone. Standard work-up and purification gives the desired products of formula I (Scheme 1).

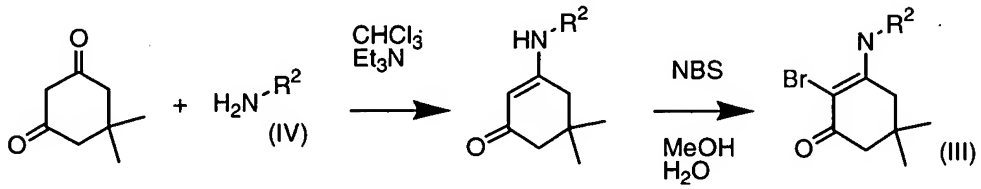
Scheme 1



Wherein $=Z^1-Z^2=Z^3-Z^4=$, R^1 and R^2 are defined as for the compounds of formula (I) hereinbefore.

The appropriate 2-bromo-5,5-dimethyl-3-oxo-cyclohex-1-enylamino derivatives (III) can generally be obtained by amination of 5,5-dimethyl-1,3-cyclohexanedione with the appropriate amine of general formula (IV) under art known amination conditions, followed by bromination with *N*-bromosuccinimide (Scheme 2).

Scheme 2

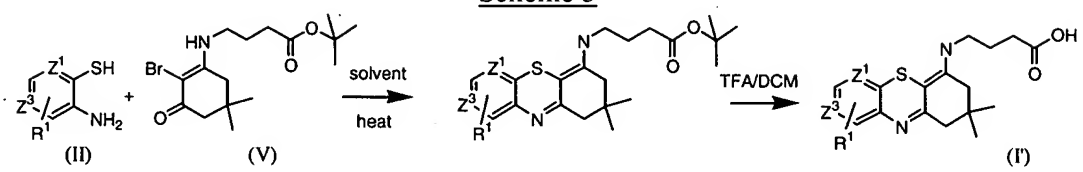


Wherein R^2 is defined as for the compounds of formula (I) hereinbefore.

For those compounds of formula (I) where R^2 represents butyric acid, hereinafter referred to as the compounds of formula (I'), the compounds are obtained by condensing the ortho-amino substituted (hetero)arene-thiol (II) with 4-(2-bromo-5,5-dimethyl-3-oxo-cyclohex-1-enylamino)-butyric acid or an ester derivative such as a t-butylester (V) using art known conditions, such as for example by heating the two reactants in a suitable solvent, such as ethanol or N-methylpyrrolidone. Standard work-up and purification gives the desired products, or the ester derivative, which can be

hydrolyzed under acidic or basic conditions to give the required butyric acids (I') (Scheme 3).

Scheme 3



Further examples for the synthesis of compounds of formula (I) using the above mentioned synthesis method is provided in the experimental part hereinafter.

Where necessary or desired, any one or more of the following further steps in any order may be performed:

(i) removing any remaining protecting group(s);

(ii) converting a compound of formula (I) or a protected form thereof into a further compound of formula (I) or a protected form thereof;

(iii) converting a compound of formula (I) or a protected form thereof into a *N*-oxide, a salt, a quaternary amine or a solvate of a compound of formula (I) or a protected form thereof;

(iv) converting a *N*-oxide, a salt, a quaternary amine or a solvate of a compound of formula (I) or a protected form thereof into a compound of formula (I) or a protected form thereof;

(v) converting a *N*-oxide, a salt, a quaternary amine or a solvate of a compound of formula (I) or a protected form thereof into another *N*-oxide, a pharmaceutically acceptable addition salt a quaternary amine or a solvate of a compound of formula (I) or a protected form thereof.

It will be appreciated by those skilled in the art that in the processes described above the functional groups of intermediate compounds may need to be blocked by protecting groups.

Functional groups which it is desirable to protect include hydroxy, amino and carboxylic acid. Suitable protecting groups for hydroxy include trialkylsilyl groups (e.g. tert-butyldimethylsilyl, tert-butyldiphenylsilyl or trimethylsilyl), benzyl and tetrahydro-pyranyl. Suitable protecting groups for amino include tert-butyloxycarbonyl or

benzyloxycarbonyl. Suitable protecting groups for carboxylic acid include $C_{(1-6)}$alkyl or benzyl esters.

The protection and deprotection of functional groups may take place before or after a reaction step.

The use of protecting groups is fully described in 'Protective Groups in Organic Synthesis' 3rd edition, T W Greene & P G M Wutz, John Wiley & Sons Inc. (June 1999).

Additionally, the N-atoms in compounds of formula (I) can be methylated by art-known methods using CH_3-I in a suitable solvent such as, for example 2-propanone, tetrahydrofuran or dimethylformamide.

Some of the intermediates and starting materials as used in the reaction procedures mentioned hereinabove are known compounds and may be commercially available or may be prepared according to art-known procedures.

Method of Treatment

The present invention also provides the use of a compound identified as a $GABA_B$ receptor activity modulator, using one of the aforementioned assays, in particular the compounds of formula (I) as described hereinbefore, in the manufacture of a medicament for the treatment an indication such as stiff man syndrome, gastroesophogeal reflux, neuropathic pain, incontinence and treatment of cough and cocaine addiction. In particular for use in the manufacture of a medicament to reduce transient lower esophagal sphincter relaxations (TLESR). It is thus an object of the present invention to provide a method for the treatment of a warm-blooded animal, for example, a mammal including humans, suffering from an indication such as stiff man syndrome, gastroesophogeal reflux, neuropathic pain, incontinence and treatment of cough and cocaine addiction, in particular TLESR.

Said method comprising administering to a warm-blooded animal in need thereof an effective amount of a compound identified as a $GABA_B$ receptor modulator using a method according to the invention. In particular the systemic or topical administration of an effective amount of a compound according to the invention, to warm-blooded animals, including humans.

Such agents may be formulated into compositions comprising an agent together with a pharmaceutically acceptable carrier or diluent. The agent may in the form of a physiologically functional derivative, such as an ester or a salt, such as an acid addition salt or basic metal salt, or an N or S oxide. Compositions may be formulated for any suitable route and means of administration. Pharmaceutically acceptable carriers or diluents include those used in formulations suitable for oral, rectal, nasal, inhalable, topical (including buccal and sublingual), vaginal or parenteral (including subcutaneous, intramuscular, intravenous, intradermal, intrathecal and epidural) administration. The choice of carrier or diluent will of course depend on the proposed route of administration, which, may depend on the agent and its therapeutic purpose. The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

For solid compositions, conventional non-toxic solid carriers include, for example, pharmaceutical grades of mannitol, lactose, cellulose, cellulose derivatives, starch, magnesium stearate, sodium saccharin, talcum, glucose, sucrose, magnesium carbonate, and the like may be used. The active compound as defined above may be formulated as suppositories using, for example, polyalkylene glycols, acetylated triglycerides and the like, as the carrier. Liquid pharmaceutically administrable compositions can, for example, be prepared by dissolving, dispersing, etc, an active compound as defined above and optional pharmaceutical adjuvants in a carrier, such as, for example, water, saline aqueous dextrose, glycerol, ethanol, and the like, to thereby form a solution or suspension. If desired, the pharmaceutical composition to be administered may also contain minor amounts of non-toxic auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like, for example, sodium acetate, sorbitan monolaurate, triethanolamine sodium acetate, sorbitan monolaurate, triethanolamine oleate, etc. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art; for example, see Gennaro et al., Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pennsylvania, 18th Edition, 1990.

The composition or formulation to be administered will, in any event, contain a quantity of the active compound(s) in an amount effective to alleviate the symptoms of the subject being treated.

Dosage forms or compositions containing active ingredient in the range of 0.25 to 95% with the balance made up from non-toxic carrier may be prepared.

For oral administration, a pharmaceutically acceptable non-toxic composition is formed by the incorporation of any of the normally employed excipients, such as, for example, pharmaceutical grades of mannitol, lactose, cellulose, cellulose derivatives, sodium crosscarmellose, starch, magnesium stearate, sodium saccharin, talcum, glucose, sucrose, magnesium, carbonate, and the like. Such compositions take the form of solutions, suspensions, tablets, pills, capsules, powders, sustained release formulations and the like. Such compositions may contain 1%-95% active ingredient, more preferably 2-50%, most preferably 5-8%.

Parenteral administration is generally characterized by injection, either subcutaneously, intramuscularly or intravenously. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like. In addition, if desired, the pharmaceutical compositions to be administered may also contain minor amounts of non-toxic auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like, such as for example, sodium acetate, sorbitan monolaurate, triethanolamine oleate, triethanolamine sodium acetate, etc.

The percentage of active compound contained in such parental compositions is highly dependent on the specific nature thereof, as well as the activity of the compound and the needs of the subject. However, percentages of active ingredient of 0.1% to 10% in solution are employable, and will be higher if the composition is a solid which will be subsequently diluted to the above percentages. Preferably, the composition will comprise 0.2-2% of the active agent in solution.

Throughout this description the terms "standard methods", "standard protocols" and "standard procedures", when used in the context of molecular biology techniques, are to be understood as protocols and procedures found in an ordinary laboratory

manual such as: Current Protocols in Molecular Biology, editors F. Ausubel et al., John Wiley and Sons, Inc. 1994, or Sambrook, J., Fritsch, E.F. and Maniatis, T., Molecular Cloning: A laboratory manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY 1989.

This invention will be better understood by reference to the Experimental Details that follow, but those skilled in the art will readily appreciate that these are only illustrative of the invention as described more fully in the claims that follow thereafter. Additionally, throughout this application, various publications are cited. The disclosure of these publications is hereby incorporated by reference into this application to describe more fully the state of the art to which this invention pertains.

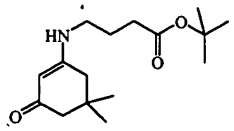
EXPERIMENTAL PART

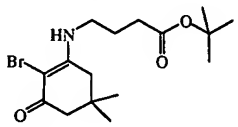
I SYNTHESIS OF $GABA_B$ AGONISTS

In the procedures described hereinafter the following abbreviations were used : "DIPE" stands for diisopropylether; "EtOAc" stands for ethyl acetate.
For some chemicals the chemical formula was used, e.g. CH_3CN for acetonitrile, NH_3 for ammonia, CH_2Cl_2 for dichloromethane, $MgSO_4$ for magnesium sulfate, and HCl for hydrochloric acid.

A. Preparation of the intermediates

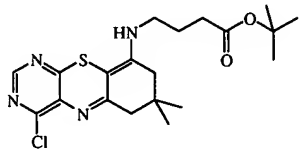
Example A.1

Preparation of  intermediate 1

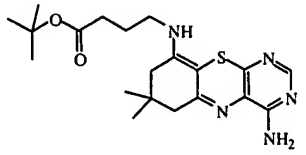
and  intermediate 2

4-Aminobutanoic acid 1,1-dimethylethyl ester [50479-22-6] (14g, 0.087mol) and 5,5-dimethyl-1,3-cyclohexanedione [126-81-8] (12.26g, 0.087mol) were dissolved in trichloromethane (250 ml) and *N,N*-diethylethanamine (0,5ml) was added. The reaction mixture was stirred for 3 days and subsequently washed with three portions of 250ml of water. The organic layer was dried on $MgSO_4$ and concentrated under reduced pressure. The residue was recrystallised in DIPE/ CH_3CN to give 18.6g (76%) of intermediate 1.

This product was taken up in methanol (250 ml) and water (100 ml). 1-Bromo-2,5-pyrrolidinedione (11.8g, 0.066 mol) was added portionwise over a 30 minutes period. After stirring for an additional hour, 500 ml water was added The mixture was extracted with three portions of dichloromethane. The combined organic layers were dried on $MgSO_4$ and concenterated under reduced pressure to yield 22g (92%) of intermediate 2.

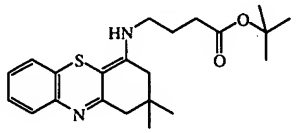
In a similar way was also prepared :  intermediate 3

Example A.2

Preparation of  intermediate 4

A mixture of 5,6-diamino-4(1*H*)-pyrimidinethione [2846-89-1](0.0027 mol) and intermediate 2 (0.0027 mol) in ethanol (q.s.) was stirred for 2 hours at 85°C. The reaction mixture was filtered and the solvent was evaporated. The residue was purified by high-performance liquid chromatography. The product fractions were collected and the solvent (CH_3CN) was evaporated. The aqueous layer was extracted with EtOAc. The organic layer was separated, dried ($MgSO_4$), filtered and the solvent was evaporated, yielding 0.400 g (30%) of intermediate 4.

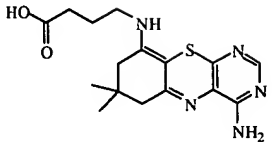
Example A.3

Preparation of  intermediate 5

A mixture of 2-aminobenzenethiol [137-07-5] (0.004 mol) and intermediate 2 (0.004 mol) in 1-methyl-2-pyrrolidinone [872-50-4] (15 ml) was stirred for 1 hour at 140°C. The reaction mixture was cooled and the layers were separated with $EtOAc/H_2O(NH_3)$. The organic layer was dried ($MgSO_4$), filtered and the solvent was evaporated. The residue was purified by high-performance liquid chromatography. The product fractions were collected and the solvent (CH_3CN) was evaporated. The aqueous layer was extracted with EtOAc and then the organic layer was dried ($MgSO_4$), filtered off and the solvent was evaporated, yielding 0.6 g (40 %) of intermediate 5.

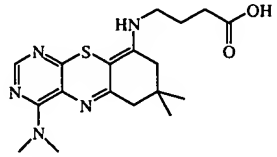
B. Preparation of the compounds

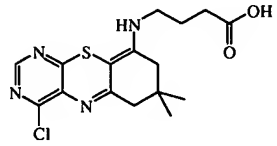
Example B.1

Preparation of  compound 2

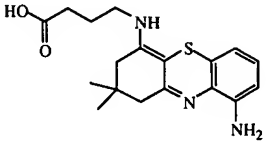
A mixture of intermediate 4 (0.001 mol) in trifluoroacetic acid (5 ml) and dichloromethane (5 ml) was stirred for 1 hour at room temperature. The reaction mixture was dried under a stream of nitrogen. The resulting residue was suspended in diethyl ether. The desired product was filtered off and dried (vacuo) at 30°C, yielding 0.120 g (23 %) of trifluoroacetic acid salt of compound 2.

In a similar way were also prepared :

The hydrobromic acid salt of  compound 1

and the trifluoroacetic acid salt of  compound 4

Example B.2

Preparation of  compound 3

A mixture of intermediate 5 (0.00155 mol) in trifluoroacetic acid (5 ml) and dichloromethane (5 ml) was stirred for 20 hours at room temperature. The reaction mixture was dried under a stream of nitrogen. The resulting residue was solidified in diethyl ether. The desired product was filtered off and dried (vacuo) at 30°C, yielding 0.320 g (67 %) of trifluoroacetic acid salt of compound 3.

II DEVELOPMENT OF $GABA_B$-CHO-K1 CELLS

MATERIAL AND METHODS

Permanent transfection of GABABR1a and GABABR2 in CHO-K1 cells using Lipofectamine PLUS:

CHO-cells were transfected with hGABABR1a/pcDNA3.1. Monoclonal stable R1a-expressing cells were transfected with hGABABR2/pcDNA3.1Hygro+. Selection of clones occurred with 800 µg geneticin + 800 µg hygromycine/ml.

Membrane preparation:

Butyrate-stimulated (5 mM final) cells were scraped, after a short rinse with PBS, in 50 mM TrisHCl pH7.4 and centrifuged at 23500 g for 10 min. at 4°C. The pellet was homogenised in 5 mM TrisHCl pH 7.4 by Ultra-Turrax (24000 rpm) followed by centrifugation at 30000 g for 20 min. at 4°C. The resulting pellet was resuspended in 50 mM TrisHCl pH 7.4 and rehomogenised. Protein concentration was determined using the Bradford method.

GTPγ35S activation assay:

10 µg membrane prep was incubated in 250 µl in 20 mM Hepes pH 7.4, 100 mM NaCl, 3 mM MgCl2, 0.25 nM GTPγ35S, 3 µM GDP, 10 µg saponin/ml, with or without 1mM GABA (basal activity in absence of baclofen) at 37°C for 20 min. Filtration was carried out onto 96-well GF/B filter plate in Harvester (Packard). Filters were rinsed 6 times with cold 10 mM phosphate buffer pH 7.4, and dried overnight before addition of 30 µl Microscint O, and measurement in Topcount (Packard, 1min./well).

3H-agonist binding:

30 - 60 µg membrane prep was incubated in 50 mM TrisHCl pH 7.4, 2.5 mM CaCl2, 10 nM 3H-GABA or 20 nM 3H-baclofen in 500 µl at 20°C. Non-specific binding was determined in the presence of 100 µM baclofen. After 90 minutes the mixture was transferred onto 96-well GF/B filterplate by Harvester (Packard). Filters were rinsed 6 times with cold 50 mM TrisHCl pH 7.4, 2.5 mM CaCl2 , and dried overnight before addition of 30 µl Microscint O, and measurement in Topcount (Packard, 1min./well).

RESULTS

GTPγ^{35}S activation assay

In membranes of stably hGABABR1a-transfected CHO-cells, we measured binding of the antagonist 3H-CGP54626. hGABABR2 was co-transfected in those R1a-clones with the highest antagonist binding. After subcloning stable clones were obtained

showing functional activity in GTPγ35S-binding assay upon stimulation of membranes by GABA, wherein said activity was potentiated in the presence of the positive modulator CGP7930 (Urwyler S., *et al.*, 2001, Molecular Pharmacology60:963-971) (fig. 1).

Agonist Filter Binding Assay

An agonist filter binding assay has been developed in 96-well GF/B filterplate. The IC50 of known agonists and antagonists was determined (fig.2). While the stable $hGABA_BR1a$ or the transient $hGABA_BR2$ monomeric $GABA_B$ receptor expressing cells did not show any binding to the agonists 3H-GABA or 3H-baclofen (data not shown), unexpectedly, in our hGABABR1a/R2 heterodimeric clone agonist binding was detected with both ligands . The Kd for 3H-baclofen, 3H-GABA, and 3H-CGP54626 was determined in saturation experiments and compared well with published results obtained with tissue preparations (table 1).

Table 1

^{3}H-baclofen		
Rat	132 nM	(Hill & Bowery, 1981)
Dog cortex	28 nM	(J&JPRD, 2000)
$hGABA_BR1aR2$/CHO	30 nM	(our data, n=2))
^{3}H-GABA		
Rat	77 nM	(Hill & Bowery, 1983)
Rat	15-30 nM	(Cross & Horton, 1988)
Pig	26 nM	(Facklam & Bowery, 1993)
Human	20-30 nM	(Cross & Horton, 1988)
$hGABA_BR1aR2$/CHO	10-30 nM	(our data, n=6)
^{3}H-CGP54626		
Rat	1.5 nM	(Bittiger et al., 1993)
Pig	1.35 nM	(Facklam & Bowery, 1993)
$hGABA_BR1aR2$/CHO	1.5 nM	(Green et al., 1993)
$hGABA_BR1aR2$/CHO	2.78 nM	(our data, n=1)

The order of potency for agonists was AMPA > GABA > baclofen, and for antagonists CGP54626 > SCH50911 (fig.2). The obtained IC50s were reproducible between different membrane preparations (fig.3)

Upon full library screening we identified some compounds with binding and signal transduction properties with comparable potencies as the reference compounds GABA and baclofen (table 2).

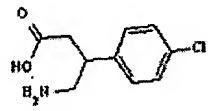
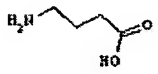
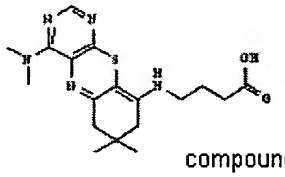
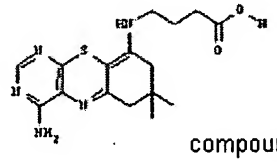
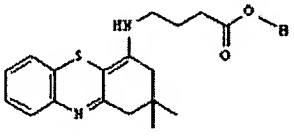
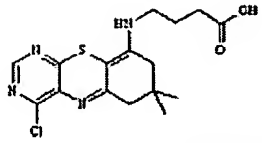
	BINDING ASSAY	SIGNAL TRANSDUCTION
	^{3}H-GABA binding	GTPγS binding
Chemistry	pIC50	%Effect at 10 µM
Reference compounds		
 baclofen	6.90775	78.7021
 GABA	8.06026	79.2906
HTS hits		
 compound 1	7.1875	45.7275
 compound 2	6.82	40.95
 compound 3	6.43	24.44
 compound 4	6.87	61.93

Table 2 : pIC_{50} and % effect in the GABA ligand binding, and GTPγS signal transduction assays for reference compounds and HTS hits.

*Agonist centrifugation Binding Assay*In an alternative binding assay the non-bound ligand was separated from the membranes by centrifugation instead of filtration. The assay was performed according to the earlier described filter binding assay, with the difference that the non-bound ligand was separated from the membranes by centrifugation in a microcentrifuge at 12500 rpm for 10 minutes. The supernatant was discarded, the pellet was rinsed with washing buffer and dissolved in 200 µl water. Scintillation fluid was added and the bound ^{3}H-GABA measured in Topcount (Packhard, 1 min./well).

In a saturation assay using increasing concentrations of ^{3}H-GABA (1 – 400 nM final) I was found that the $GABA_B$ receptor expressed by the $hGABA_BR1a/GABA_BR2$ CHO cell line, possess a low and a high affinity agonist binding site. Results of the saturation and scatchard analysis are summarized in Table 3. When the saturation assay was performed in the presence of 10 µM of the $GABA_B$ antagonist CGP54626 or one of the $GABA_B$ agonist of the present invention (compound 1), the ^{3}H-GABA binding to both the high and the low affinity site was blocked (figure 4a, b).

Table 3

	Mean (n=5) **nM**	**SD** **nM**
Bmax 1	0.19	0.05
Kd 1	9.4	3.1
Bmax 2	0.76	0.24
Kd 2	401	224

DISCUSSION

To our knowledge, no earlier reports were made in literature of recombinant $hGABA_B$ receptor, showing agonist binding with a high and low affinity binding site in a filter binding assay. An HTS agonist filter binding screen has been developed using 3H-GABA. We found reproducible Ki values for known agonists and antagonists, independent of the membrane preparation.

It has in addition been demonstrated that the recombinant $GABA_B$ receptor has two agonist binding sites. One high affinity and one low affinity binding site. It is to be expected that high affinity agonists of the $GABA_B$ receptor will ellict a different response compared to the low affinity agonists. Hence, the cell line of the present invention not only allows to identify $GABA_B$ receptor agonists, but also provides a useful tool to characterize the nature of the compound receptor interaction.